Effect of Biotic Elicitors on Enrichment of Antioxidant Properties and Induced Isoflavones in Soybean

S.M. Boué, F.F. Shih, B.Y. Shih, K.W. Daigle, C.H. Carter-Wientjes, and T.E. Cleveland

ABSTRACT: The antioxidant properties of methanolic extracts from soybean obtained with germination, wounding, and application of biotic elicitors were evaluated. Also, the relationship between observed antioxidant properties and compositional changes in isoflavone content was determined. The 2 biotic elicitors used in this study were the food-grade fungus *Aspergillus sojae* and *A. sojae* cell wall extract. Isoflavone content was determined by C_{18} reverse phase high-performance chromatography coupled with a photodiode array detector. Antioxidant activities of the extracts were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and β-carotene cooxidation in a linoleate system. Higher antioxidant activities were observed in wounded and elicitor-treated extracts when compared with nonwounded control extracts. In addition, the phenolic content was higher in extracts from wounded and elicitor-treated soybean. Germination for 3 d slightly decreased total isoflavone content (–4.3%); however, wounding increased total isoflavone content (25.8%). The soybean extracts from seeds treated with *A. sojae* biotic elicitors had the highest total isoflavone contents (9.8 to 11.6 mg/g extract) and displayed the highest antioxidant activities in both the DPPH and β-carotene assays. Also identified in the wounded and elicitor-treated extracts were the induced isoflavones glyceollins that contributed to the higher isoflavone contents observed.

Keywords: antioxidant, elicitor, isoflavone, glyceollins, soybean

Introduction

Soybean (*Glycine max*) consumption has been linked to many health-promoting activities, including reduced risk of various cancers (Adlercreutz 1990; Wu and others 1996; Fournier and others 1998; Messina and Loprinzi 2001) and coronary heart disease (Diplock 1994; Rice-Evans and others 1996). Many of the health benefits of soybean have been attributed, in part, to the presence of isoflavones (Ruiz-Larrea and others 1997; Lee and others 2004, 2005). Isoflavones act as anticancer agents through several different mechanisms, but researchers have postulated that part of the reason is the ability of isoflavones to function as antioxidants. Isoflavones have been shown to inhibit free radical formation (Ruiz-Larrea and others 1997; Lee and others 2004, 2005), reduce lipid oxidation (Kapiotis and others 1997), and stimulate antioxidant enzymes (Kameoka and others 1999). The ability of isoflavones to act as antioxidants is based on the fact that they are able to form delocalized unpaired electrons, stabilizing the formed phenoxyl radical after reaction with lipid radicals (Gordon 1990). Isoflavones found in soybean are in the aglycone, β-glucoside, 6-O"-malonyl-β-glucoside, or 6-O"-acetyl-β-glucoside forms (Barnes and others 1994; Wang and Murphy 1996; Wang and others 1998; Murphy and others 2002; Lee and others 2004). Raw soybeans contain predominately the glucoside forms of the isoflavones and a low percentage of the aglycone form. Many of the health-promoting activities of isoflavones have been attributed to the aglycone form of isoflavones; however, the glucoside forms also possess antioxidant activity (Lee and others 2005).

Several factors can alter soybean isoflavone concentrations. Isoflavone concentrations have been shown to change during germination. Kim and others (2004) reported significant increases in the aglycones daidzein and genistein during the 1st 8 d of germination and only slight increases in glycosides. In addition, several significant changes in isoflavone concentration occur in response to stress or elicitor treatment. Soybean cotyledons treated with *Phytophthora megasperma* f. sp. *glycinea* wall glucan contained higher concentrations of both isoflavone aglycones and glycosides (Graham and Graham 1991). In response to stress, soybean produces the phytoalexins glyceollins I, II, and III that are derived from the isoflavone daidzein (Darvill and Albersheim 1984; Graham and others 1990; Graham and Graham 1991; Paxton 1991). The glyceollins are low molecular weight antimicrobial compounds that are synthesized *de novo* and accumulate in different soybean tissues. The glyceollins are lipophilic isoflavones that are products of secondary metabolism and often accumulate at infection sites in concentrations that inhibit fungal and bacterial growth. Countless stress factors or physical stimuli induce the glyceollins accumulation, including wounding, freezing, ultraviolet light exposure, and exposure to microorganisms (Darvill and Albersheim 1984; Graham and others 1990; Graham and Graham 1991; Paxton 1991). Although the glyceollins are detected at high concentrations in soybeans during stress, they have also been detected at trace levels in nonelicitor treated soybean seeds (Kraus and others 1995).

Research in our laboratory has focused on the antiestrogenic activity of the glyceollins *in vitro* (Burow and others 2001) and *in vivo* (Salvo and others 2006; Wood and others 2006). In contrast to the estrogenic effects of the constitutive soy isoflavones daidzein and genistein, the glyceollins displayed a marked antiestrogenic effect on estrogen receptor signaling, which correlated with a comparable suppression of estrogen-induced proliferation of breast cancer cells. This antiestrogenic activity was also observed

MS 20070710 Submitted 9/14/2007 Accepted 1/28/2008. *Authors are with U.S. Dept. of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA 70179, U.S.A. Direct inquiries to author Boué (E-mail: sboue@ srrc.ars.usda.gov).*

doi: 10.1111/j.1750-3841.2008.00707.x

in an animal model system. Treatment with glyceollin suppressed estrogen-stimulated tumor growth of breast and ovarian cancer cells in overiectomized athymic mice (Salvo and others 2006). A 2nd animal study with female postmenopausal cynomolgus macaques was conducted to test the effects of a glyceollin-enriched soy protein isolate produced from stressed soybeans on estrogen exposure (Wood and others 2006). The treatment group receiving estrogen along with the glyceollin-enriched soy protein displayed no significant increase in breast proliferation. These results suggested that glyceollin-enriched soy inhibited the harmful effects of estrogen and further investigation of the glyceollin's effect on health is warranted, including their ability to function as antioxidants.

Very little data are available on the antioxidant activity of extracts obtained from germinating and elicitor-treated soybean seeds. In the present study, we evaluated the antioxidant activity of several soybean extracts obtained under varying conditions using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and the β-carotene cooxidation methods. The DPPH method has been widely used to evaluate the free radical scavenging effectiveness of plant extracts. The rate of bleaching of β-carotene is also a widely used method to determine antioxidant activity. The antioxidant activities of soybean extracts obtained from germinating seeds were compared to raw (dry) seeds to test the hypothesis that germination alters phenolic content and antioxidant activity. Extracts containing the induced isoflavone glyceollins were also obtained from wounded seeds and seeds exposed to both the live fungus and a fungal cell wall extract from *Aspergillus sojae*. The present study was conducted to evaluate total phenolics and antioxidant activities of induced soybean extracts for their possible utilization in foods or functional foods or pharmaceutical supplements.

Materials and Methods

Materials

Seeds from the soybean (*Glycine max* L.) culitvar Asgrow 5902 were obtained from Helena Chemical Co. (Thibodaux, La., U.S.A.). *Aspergillus sojae* (SRRC 1125) cultures were obtained and grown at the Southern Regional Research Center, USDA, New Orleans, La., U.S.A.

Chemicals

HPLC-grade methanol, acetonitrile, and water were used as solvents for the study and were purchased from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Genistein, genistin, daidzein, daidzin, glycitein, and glycitin were obtained from Indofine Chemical Co. (Somerville, N.J., U.S.A.). The glyceollins used as a standard for quantitation were isolated using a procedure developed at the Southern Regional Research Center, USDA (Burow and others 2001). Soybean seeds (200 g) were sliced and inoculated with *A. sojae*. After 3 d, glyceollins were extracted from the inoculated seeds with 100% methanol. The glyceollins (mixture of glyceollins I, II, and III in a ratio of 6:2:1) were isolated using preparative-scale HPLC and confirmed by UV-VIS spectrophotometry, electrospray mass spectrometry, and NMR. The solvents acetonitrile (HPLC grade) and methanol were purchased from Aldrich Chemical Co. (Milwaukee, Wisc., U.S.A.). H_2O treated with a Millipore system was used during sample preparation procedures and HPLC analyses. 2,2-diphenyl-1-picrylhydrazyl, β-carotene, linoleic acid, and Folin–Ciocalteu reagent were purchased from Sigma (St. Louis, Mo., U.S.A.)

Soybean treatments

Aspergillus sojae (SRRC 1125) cultures were grown at 25 °C in the dark on potato dextrose agar. After 5 d, inoculum was prepared by harvesting conidia (3.4×10^7/mL) in 15 mL sterile, distilled H_2O. For the preparation of the cell wall extract, the method of Zeringue (1984) was used with slight modification. *A. sojae* spores were inoculated into the defined medium of Adye and Mateles contained in 500 mL culture flasks. The inoculated media was kept at 29 °C with shaking for 4 d. Mycelia were washed with distilled water, homogenized with 0.1 M phosphate buffer, filtered, defatted with chloroform–methanol (1:1), washed with ethyl ether, and air dried. Mycelia were resuspended in water, autoclaved for 3 h at 121 °C, and filtered, and the filtrate volume was concentrated by vacuum distillation. Next the concentrated filtrate was dialyzed against water at 2 °C, and then lyophilized. A cell wall extract (CWE) solution was prepared using 1 g lypholized CWE in 100 mL water.

Soybean seeds were surface-sterilized for 3 min in 70% ethanol followed by a quick deionized-H_2O rinse and two 2-min rinses in deionized-H_2O. Seeds were soaked in sterile deionized H_2O for 4 h prior to placement into Petri dishes. Each Petri dish (100 × 15 mm) was lined with 1 autoclaved filter paper (Whatman) moistened with 0.5 mL distilled H_2O. Seeds were wounded by cutting with a sterile razor longitudinally along the length of the seeds. *A. sojae* spore suspension (10 μL) and cell wall extract (10 μL) were applied to the cut surface of each seed for elicitor treatments. All chambers were stored at 25 °C in the dark for 3 d, and then transferred to –80 °C. Extracts were prepared from each seed treatment.

Extract preparation

Approximately, 6 g of seed material from treatments were lyophilized, ground, and defatted with 90 mL hexane in a Soxhlet apparatus. In some cases, a small radicle (1 to 4 mm) formed during germination and elicitor treatments, but the whole seedling was ground during sample preparation. The fat-free residue was air-dried. The antioxidant components were extracted from a 5-g aliquot of this material using 80% methanol. Each residue was extracted twice with 50 mL 80% methanol for 1 h by ultrasonic treatment. The combined extracts were filtered, vacuum-evaporated to remove methanol, and freeze-dried. The dried material was weighed to determine extraction yield, and stored at –20 °C until use. The dried material (125 mg) was dissolved in 15 mL methanol for antioxidant and HPLC analyses.

Determination of total polyphenolic compounds

The amount of total polyphenolic compounds was measured by a method described by Taga and others (1984). In brief, about 125 mg of freeze-dried extract were dissolved in 15 mL methanol, and 2 mL of extract solution were filled up with 0.3% HCl to 5 mL. A 100-μL aliquot of Folin–Ciocalteau reagent (diluted with ethanol 1:1) was added. After 30 min, the absorbance was measured at 765 nm using a Shimadzu 160A spectrophotometer (Shimadzu, Houston, Tex., U.S.A.). The concentration was calculated using gallic acid as standard, and the results expressed as milligrams gallic acid equivalents (GAE) per gram extract. Samples were measured in triplicate.

Determination of antioxidant activity with the DPPH radical scavenging method

To determine antioxidant activity of the soybean extracts, the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used following the method described by Matthäus (2002). Radical scavenging activity was measured by a decrease in the absorbance of DPPH at 515 nm. For each extract, different concentrations were tested. An aliquot (0.5 mL) of the DPPH solution (50 mg/100 mL) was diluted in 4.5 mL of methanol, and 0.1 mL of a methanolic solution of the extract was added. The mixture was shaken vigorously and

allowed to stand in the dark for 45 min. The decrease in absorbance was measured at 515 nm against a blank with a spectrophotometer. From a calibration curve obtained with different amounts of extract, the ED_{50} (amount of extract required to quench 50% of the initial DPPH radicals) was calculated.

Determination of antioxidant activity with the β-carotene bleaching method

Determination of antioxidant activity using a β-carotene/linoleic acid system was carried out according to the method described by Marco (1968). A β-carotene solution was prepared by dissolving 10 mg of the reagent in 200 mL chloroform. Two grams of Tween 40 and 250 mg linoleic acid were added to 30 mL of the β-carotene solution. An aliquot (3 mL) of the resulting mixture was subjected to a stream of nitrogen gas to remove the chloroform. After evaporation, 25 mL oxygenated distilled water were added and the mixture was shaken to form a liposome solution. After 3 min, 3 mL of the emulsified solution were added 100 μL sample extract. After a 2-min equilibration period in a cuvette, the solution was read at 470 nm every 30 s for 15 min on a spectrophotometer with the temperature of the chamber maintained at 50 °C. Antioxidant activity was calculated as percent inhibition (percent reduction in bleaching rate) relative to control using the following equation:

$$\%\ \text{antioxidant activity} = [(R_{\text{control}} - R_{\text{sample}})/R_{\text{control}}] \times 100$$

where R_{control} and R_{sample} were the bleaching rates of β-carotene in a reactant mix with the control and plant extract, respectively.

HPLC analysis

HPLC analyses were performed on a Waters 2695 combined with a Waters UV-VIS 2996 photodiode array detector (PDA) (Waters Assoc., Milford, Mass., U.S.A.). Isoflavones were separated using a Phenomenex Luna II C_{18} reverse-phase column (4.6 × 250 mm; 5 μm). A guard column containing the same packing was used to protect the analytical column. The injection volume of sample was 20 μL with a flow rate of 1.0 mL/min with the following solvent system: A = acetic acid/water (pH = 3.0), B = acetonitrile; 15% B to 55% B in 58 min, then 55% B to 100% B in 16 min followed by holding at 100% B for 6 min. The spectra were collected between 220 and 400 nm by PDA, and compounds were quantified at 260 (isoflavones) and 285 nm (glyceollins).

Standard curves

Standard curves were prepared for genistein, genistin, daizdein, daidzin, glycitein, glycitin, and glyceollin. Approximately 1 mg of each standard compound was dissolved in 80% methanol in water (100 mL) to prepare stock solutions. A small volume of DMSO (20 μL) was used to dissolve standards initially to improve solubility. Each stock solution was serially diluted with 80% methanol in water. Each isoflavone standard solution was injected into the HPLC, and the relationship between peak area and concentration from the UV-vis spectrophotometer was calculated. Although standards for the malonyl-β-glycosides of genistin, daidzin, and glycitin were not available, research by Kudou and others (1991) has shown that the molar extinction coefficient of the malonyl conjugate approximates that of the β-glucoside. Therefore, the standard curves of genistin, daidzin, and glycitin were used for quantifying the malonyl conjugates. Isoflavone concentration was expressed as μg/g dried extract.

Statistical analysis

Results are presented as mean value ± standard deviation. Statistical comparisons were made by analysis of variance (ANOVA) procedure followed by a Duncan's multiple range tests. $P < 0.05$ was considered significantly different.

Results and Discussion

Amount of extractable compounds compared with extractable phenolic compounds

The effect of germination, wounding, and biotic elicitors on extractable compounds and total phenolics is shown in Table 1. The amount of extractable compounds was highest for the unwounded (dry and germinated) soybean extracts. The extractable compound amounts ranged from 148.7 mg/g for the germinated seed to 121.2 mg/g for the germinated, cut, cell wall extract (CWE)-treated seed. The amount of total phenolic compounds in the extracts was determined by the Folin–Ciocalteau assay. Table 1 displays the results of this assay expressed as gallic acid equivalents. The extracts contained between 11.3 and 22.0 mg of phenolic compounds per gram extract. Total phenolic content was increased to 15.3 mg/g when seeds were treated with the *A. sojae* cell wall extract. However, the highest level (22.0 mg/g) was obtained from the cut, *A. sojae*-treated extract. The dry seed extract contained the lowest level (11.3 mg/g) of total phenolic compounds. Soaking the seeds in water and allowing them to germinate for 3 d increased the total phenolic content only slightly to 11.5 mg/g. However, germination with wounding increased the phenolic content to 14.2 mg/g.

The ratio of total phenolic compounds to the total extractable compounds ranged from 7.7% to 17.6% (Table 1). This meant that in all cases between 82.4% and 92.3% of the extractable compounds were other than phenolic compounds. The lowest ratio was obtained for the germinated seed extract (7.7%). A higher ratio was obtained for the germinated, cut seed extract (10.9%). Treatment with fungal CWE increased the ratio to 12.6%, and treatment with *A. sojae* fungi increased the ratio to 17.6%. Wounding and treatment with elicitor significantly increased the ratio of total phenolic compounds to the total extractable compounds.

Isoflavone analysis and effects of biotic elicitors

Genistein, daidzein, and glycitein (see Figure 1) as well as their β-glucosides (genistin, daidzin, and glycitin) and 6-O"-malonyl-β-glucosides (malonylgenistin, malonyldaidzin, and malonylglycitin) were successfully identified and quantified using HPLC. Also identified in treatments involving wounding and elicitors were the induced glyceollins (combined glyceollins I, II, and III; see Figure 1). Figure 2 displays a representative HPLC chromatogram of the soybean extract derived after treatment with *A. sojae*. Soybean isoflavone content in dry and elicitor treatments is presented in Table 2. Total isoflavone content, including glyceollin concentration, increased with elicitor treatment. Soaking seeds in water and allowing them to germinate for 3 d decreased total isoflavone content

Table 1—Total extractable compounds (EC; mg/g extract) and total phenolic compounds (PC; mg gallic acid equivalents/g extract) obtained from soybean seed treatments.

Sample	EC (mg/g)	PC (mg/g)	PC/EC (%)
Dry	142.5	11.3	7.9
Germinated	148.7	11.5	7.7
Cut, germinated	130.0	14.2	10.9
Cut, CWE[a]	121.2	15.3	12.6
Cut, *fungi*[b]	125.2	22.0	17.6

[a] CWE, cell wall extract prepared from *A. sojae*.
[b] *Aspergillus sojae* fungi.

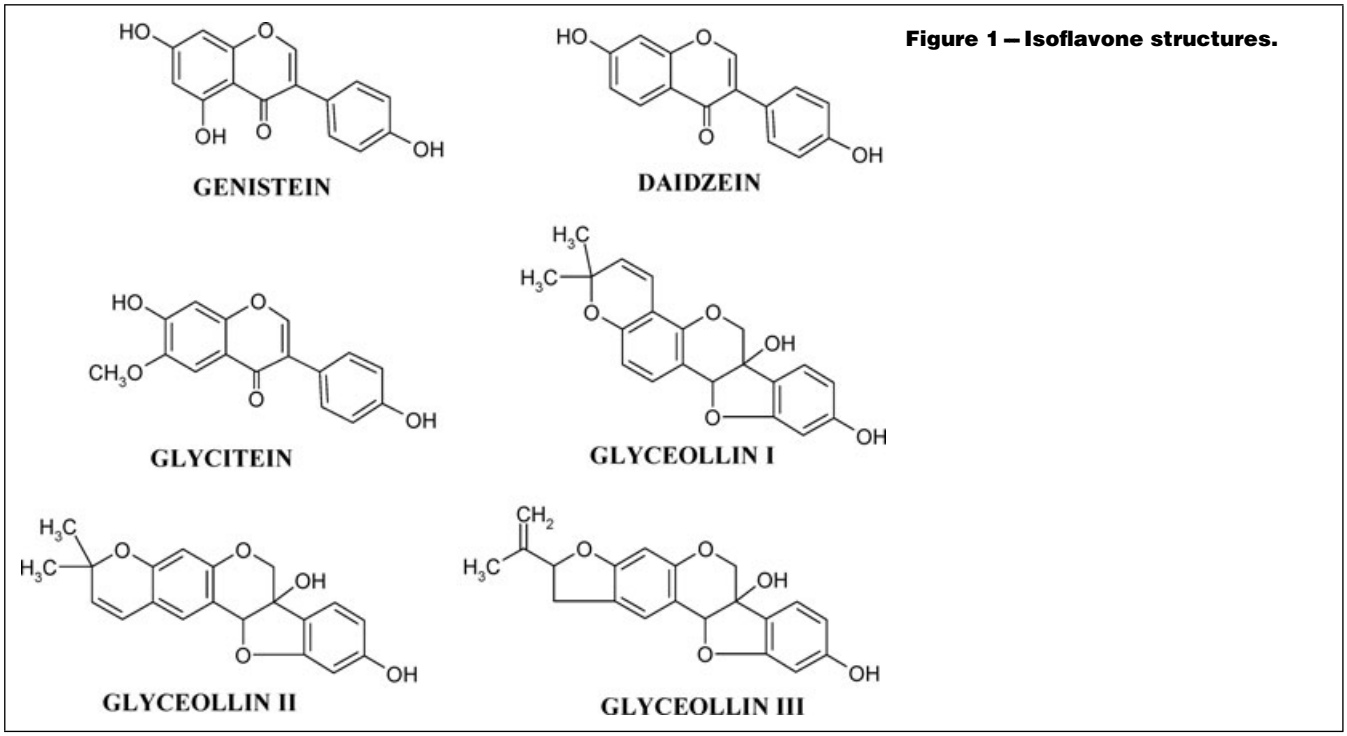

Figure 1—Isoflavone structures.

slightly (–4.3%). Soaking seeds and then wounding increased the total isoflavone content 25.8%. Glyceollin was detected in wounded seeds at low levels in this group (320 μg/g). Further treatment with a cell wall extract prepared from *A. sojae* significantly increased total isoflavone content 62.6%, and increased the glyceollin concentration to 1317 μg/g. The highest glyceollin concentration (1575 μg/g) was observed in seeds treated with the live fungus *A. sojae*. Treatment with *A. sojae* also significantly increased the total isoflavone content (93.1%) to 11.6 mg/g.

Several studies have shown that isoflavones have antioxidant activity (Gordon 1990; Rice-Evans and others 1996; Kapiotis and others 1997; Ruiz-Larrea and others 1997; Kameoka and others 1999; Lee and others 2004, 2005), and experiments have been conducted to examine the effect of increasing isoflavone concentrations on antioxidant activity (Lin and Lai 2006). Several factors may influence isoflavone content and composition, including soybean variety, growing conditions, and stage of soybean growth (germination). Establishment of a soybean seedling occurs following germination and its growth is supported by metabolites produced by hydrolysis and conversion of major stored reserves, proteins, carbohydrates, and oils (Liu 1997). In several studies, the total isoflavone content increased after 1 d of germination (Kim and others 2004; Zhu and others 2005; Lin and Lai 2006). Daidzein, genistein, glycitein, and their respective conjugates are the major soluble isoflavones in seedlings, roots, hypocotyls, and cotyledons during germination.

Other factors may also cause an increase in isoflavone content. Soybean seeds (and other soybean tissues) respond to a number of external stimuli, including wounding, pathogen attack, and elicitor treatment (Darvill and Albersheim 1984; Graham and others 1990; Graham and Graham 1991; Paxton 1991; Al-Tawaha and others 2005). Damage to the soybean plant caused by insect feeding or other processes influences changes in the isoflavone composition of the soybean seed. Wounding increases production of chemical defenses and in particularly induces the isoflavone glyceollins. Insect damaged seeds have been found to contain higher concentrations of constitutive isoflavones, including isoflavone glycosides

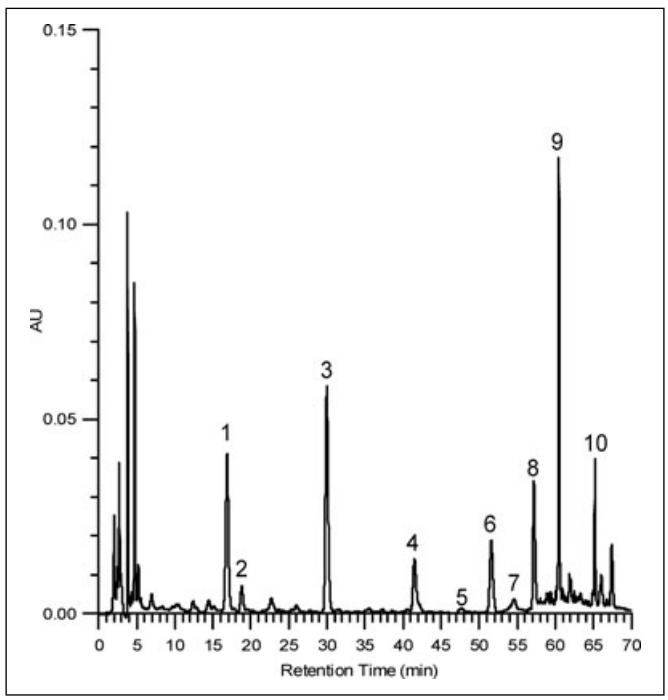

Figure 2—HPLC chromatogram of soybean seeds after treatment with *A. sojae*. 1, Daidzin; 2, glycitin; 3, genistin; 4, malonyldaidzin; 5, malonylglycitin; 6, daidzein; 7, glycitein; 8, malonylgenistin; 9, genistein; 10, glyceollins (I, II, and III).

(Piubelli and others 2003). Graham and others (1990) and Graham and Graham (1991) determined that the preexisting conjugates of both daidzein and genistein are rapidly hydrolyzed in cotyledon tissues infected by a fungal pathogen, whereby large quantities of free daidzein and genistein are released. The free daidzein may play a role as a precursor for subsequent accumulation of the phytoalexin glyceollins. Increased concentrations of isoflavone

Table 2—Isoflavone contents of seed extracts from soybean using different treatments (μg/g of extract).[a] Total isoflavones = β-glucosides (daidzin, genistin, glycitin) + malonylglucosides (MDI, MGI, MGYI) + aglycones (daidzein, genistein, glycitein) + glyceollins.[b]

Cultivar-treatment	β-glucosides			Malonyl-β-glucosides			Aglycones			Phytoalexins	
	Daidzin	Genistin	Glycitin	MDI	MGI	MGYI	Daidzein	Genistein	Glycitein	Glyceollins	Total Isoflavones
Dry	1183 ± 407	2089 ± 90	701 ± 23	621 ± 31	1187 ± 45	18 ± 9	79 ± 28	111 ± 15	33 ± 4	ND[d]	6022
Germinated	1104 ± 38	1927 ± 86	717 ± 22	582 ± 85	1087 ± 59	35 ± 10	94 ± 10	129 ± 39	85 ± 7	ND	5760
Cut, germinated	1897 ± 154	2292 ± 52	620 ± 49	510 ± 30	1104 ± 35	17 ± 3	201 ± 24	296 ± 21	218 ± 24	320 ± 13	7575
Cut, CWE[b]	2368 ± 160	2568 ± 24	615 ± 53	1151 ± 41	1157 ± 49	34 ± 4	156 ± 27	312 ± 32	115 ± 15	1317 ± 44	9793
Cut, fungi[c]	2308 ± 137	2391 ± 50	525 ± 33	963 ± 27	1132 ± 43	41 ± 5	1143 ± 59	1449 ± 87	100 ± 7	1575 ± 34	11627

[a]All samples were quantified in triplicate. Data are the means ± standard deviations.
[b]Glyceollins = total glyceollins (I, II, and III).
[c]*Aspergillus sojae* fungi.
CWE = cell wall extract; MDI = malonyldaidzin; MGI = malonylgenistin; MGYI = malonylglycitin; ND = not detected.

conjugates and aglycones were observed in cotyledon treatments using a fungal cell wall glucan after 48 h (Graham and others 1990; Graham and Graham 1991). In our study, the highest levels of conjugated and aglycone forms of isoflavones were observed in soybean seeds treated with *A. sojae* fungus. The glyceollins were observed in seeds when wounded, and the glyceollins were highest in concentration in the extracts when seeds were treated with fungal cell wall extract or the live fungus.

Antioxidant activities of soybean extracts

Two different methods have been used to determine antioxidant properties of extracts isolated from different treatments of the 3 soybean varieties: DPPH free radical scavenging and the inhibition of β-carotene cooxidation in a linoleate model system. Determination of scavenging stable free radicals is a very fast method to evaluate the antioxidant activity. Figure 3 shows the free radical scavenging activities of the soybean treatments calculated using the amount of extract required to quench 50% of the initial DPPH radicals. In this study, the amount of extract required (ED_{50}) decreased with each successive treatment. The lowest activity was obtained from the dry seed extract with an $ED_{50} = 18.6$ mg. The highest activity was obtained using the extract obtained from seeds treated with *A. sojae* fungus (10.1 mg extract) significantly lowering the ED_{50} 46% compared with the dry seed extract.

The decrease in absorbance of β-carotene in the presence of different methanolic extracts with oxidation of β-carotene and linoleic acid was used to calculate the antioxidant activities of each extract. The antioxidant activities are shown in Figure 4. Seeds treated with

Figure 3—Effect of soybean extracts from seed treatments with and without biotic elicitors on DPPH free radicals (expressed as mg extract allowing reduction of 50% DPPH). Values are means ± SD of triplicate assays. Different letters indicate significant difference ($P < 0.05$).

A. sojae cell wall extract and live fungus showed the highest antioxidant activities at 89% and 88% reduction in bleaching, respectively. Lower antioxidant activities were observed in dry (66%), germinated (65%), and germinated-cut (71%) seed treatments.

Soybean isoflavones have been extensively studied because of their potential to promote human health. One process that may make isoflavones useful is the growing evidence that shows they are antioxidants and free radical scavengers (Naim and others 1976; Mitchell and others 1998; Liebler and others 2001; Rimbach and others 2003; Lee and others 2004, 2005; Variyar and others 2004; Lin and Lai 2006). Naim and others (1976) observed that the number of hydroxyl groups in the isoflavone nucleus positively correlated with antioxidative capacity. Research by Lee and others (2005) demonstrated that the aglycone forms possess similar antioxidant activities to their corresponding conjugated forms as shown in 2 assays (ferric reducing-antioxidant power and anti-DPPH assays). However, using a low-density lipoprotein assay, they found the antioxidant potency of the glycosides was much weaker than the corresponding aglycones. Higher levels of isoflavone aglycones have been observed in fermented soybean foods, which contributed to higher antioxidant activities (Esaki and others 1999; Iwai and others 2002; Pyo and others 2005); however, in each study the soybean seeds were steamed at high temperatures, which prevented the production of the induced glyceollins during the fermentation process. High temperatures irreversibly denature practically all enzyme proteins within the seed, including those in the isoflavonoid pathway and lyse cells disrupting the biochemical signaling necessary for glyceollin production. However, research by Seshime and others (2005) has shown that *Aspergillus oryzae* possesses 4 chalcone synthase-like genes leading to the existence of flavonoid-like methabolic activity. Further research is needed to determine whether these fungal genes participate in the synthesis of plant isoflavones, including the glyceollins. In our study, an increase in isoflavone aglycones and the induced glyceollins was observed in elicitor-treated soybean extracts. Therefore, the increase of antioxidant capacity in elicitor-treated extracts might partially be caused by these increased levels of isoflavones.

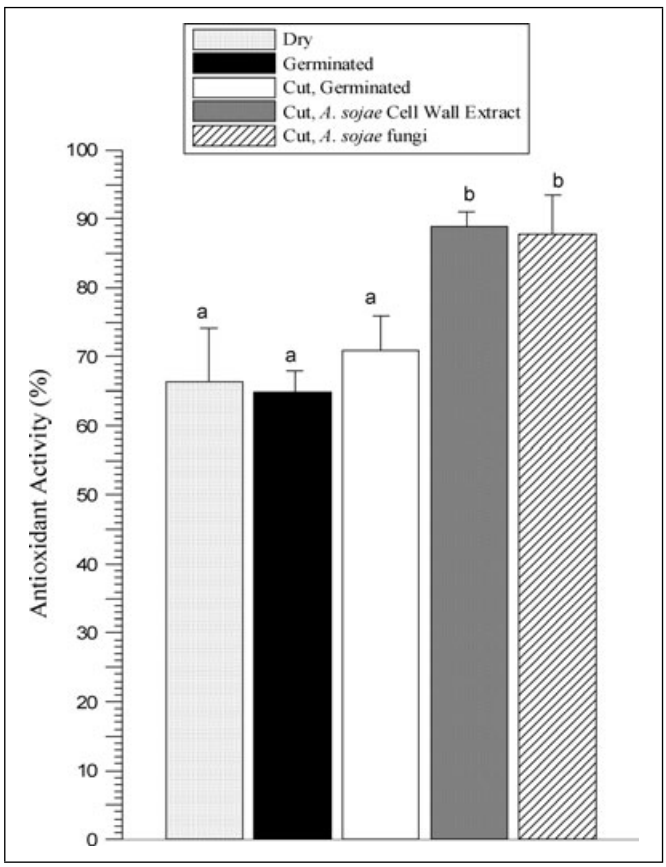

Figure 4—Effect of soybean extracts from seed treatments with and without biotic elicitors on a β-carotene/linoleic acid system. Values are means ± SD of triplicate assays. Different letters indicate significant difference ($P < 0.05$).

Soybeans contain several naturally occurring compounds other than isoflavones that have been shown to have lipid antioxidant properties that contribute to antioxidant activity (Gordon 1990; Rice-Evans and others 1996; Kapiotis and others 1997; Ruiz-Larrea and others 1997; Kameoka and others 1999; Lee and others 2004, 2005). Phenolic acids identified in soybean include syringic, vanillic, caffeic, ferulic, p-couramic, and p-hydroxybenzoic acids (Arai and others 1966). Pratt and Birac (1979) determined that besides isoflavones the following phenolic acids contributed to antioxidant activity: chlorogenic acid isomers, caffeic acid, and ferulic acid. During germination, the ascorbic acid content in soybeans increases (Bates and Matthews 1975). All of these compounds may contribute to higher antioxidant activities observed in germinated, wounded, and elicitor-treated soybean extracts.

Relationships between phenolic content, isoflavone content, and antioxidant activity

The total phenolic content of the soybean seed treatments examined in this study varied from 11.3 to 22.0 mg/g extract (Table 1). Correlation coefficients were calculated using the antioxidant activity results obtained from the 2 different methods. Using the ED_{50} values for radical scavenging activities and the total phenolic values, a correlation coefficient between these parameters was $r = -0.9783$, indicating a high negative correlation between ED_{50} values of seed treatments and the content of phenolic compounds. However, this means that there is a strong correlation between antioxidant activity and total phenolics since a lower ED_{50} value corresponds to higher antioxidant activity. Also, antioxidant activities obtained from the β-carotene method were used to determine if there was a relationship with total phenolics. The correlation coefficient between these parameters was $r = 0.8105$. These results indicate a correlation between total phenolic content and antioxidant activity. A correlation was also observed between total isoflavone content (including the glyceollins) and antioxidant activity. Using the ED_{50} values for radical scavenging activities and the total isoflavone content values, a correlation coefficient between these parameters was $r = -0.9252$, indicating a high correlation between antioxidant properties of seed treatments and total isoflavone content. Also, a high degree of correlation was determined between isoflavone content and antioxidant activity obtained from the β-carotene method ($r = 0.9521$).

Conclusions

The antioxidant activities of soybean extracts obtained from varying treatments were compared. The present study indicates that higher antioxidant activities were obtained with wounded and elicitor-treated soybean extracts when compared with nonwounded control extracts. In addition, we found higher phenolic content with wounded and elicitor-treated soybean extracts. Higher phenolic content correlated with the higher antioxidant activities determined for wounded and elicitor-treated extracts. Germination for 3 d slightly decreased total isoflavone content (–4.3%); however, wounding significantly increased total isoflavone content (25.8%). Higher isoflavone content in wounded

and elicitor-treated extracts correlated with higher antioxidant activities in the DPPH and β-carotene assays. This higher antioxidant activity is partially due to the higher concentrations of aglycones detected in elicitor treated extracts. Also identified in the wounded and elicitor treated extracts were the phytoalexin glyceollins that contributed to the higher isoflavone contents observed.

References

Adlercreutz H. 1990. Western diet and western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest 50:3–23.

Al-Tawaha AM, Seguin P, Smith DL, Beaulieu C. 2005. Biotic elicitors as a means of increasing isoflavone concentration of soybean seeds. Ann Appl Biol 146: 303–10.

Arai S, Suzuki H, Fujimaki M, Sakurai Y. 1966. Studies on flavor components in soybeans. Part 2. Phenolic acids in defatted soybean flour. Agric Biol Chem 30: 364–6.

Barnes S, Kirk M, Coward L. 1994. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. J Agric Food Chem 42:2466–74.

Bates RP, Matthews RF. 1975. Ascorbic acid and β-carotene in soybeans as influenced by maturity, sprouting, processing and storage. Proc Fl State Hort 88: 266–71.

Burow ME, Boué SM, Collins-Burow BM, Melnik LI, Duong BN, Carter-Wientjes CH, Li S, Wiese TE, Cleveland TE, McLachlan JA. 2001. Phytochemical glyceollins, isolated from soy, mediate antihormonal effects through estrogen receptor α and β. J Clin Endocrinol Metab 86:1750–8.

Darvill AG, Albersheim P. 1984. Phytoalexins and their elicitors—a defense against microbial infection in plants. Ann Rev Plant Physiol 35:243–75.

Diplock AT. 1994. Antioxidants and disease prevention. Mol Aspects Med 15:293–376.

Esaki H, Watanabe R, Onozaki H, Kawakishi S, Osawa T. 1999. Formation mechanism for potent antioxidative o-dihydroxyisoflavones in soybean fermented with *Aspergillus saitoi*. Biosci Biotechnol Biochem 63:851–8.

Fournier DB, Erdman JW, Gordon GB. 1998. Soy, its components, and cancer prevention: a review of the in vitro, animal and human data. Cancer Epi Biomarkers Prev 7:1055–65.

Gordon MH. 1990. The mechanism of antioxidant action in vitro. In: Hudson BJF, editor. Food antioxidants. London, U.K.: Elsevier Applied Science. p 1–18.

Graham TL, Graham MY. 1991. Glyceollin elicitors induce major but distinctly different shifts in isoflavonoid metabolism in proximal and distal soybean cell populations. Mol Plant-Microbe Interact 4:60–8.

Graham TL, Kim JE, Graham MY. 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. Mol Plant-Microbe Interact 3:157–66.

Iwai K, Nakaya N, Kawasaki Y, Matsue H. 2002. Antioxidative functions of natto, a kind of fermented soybeans: effect on LDL oxidation and lipid metabolism in cholesterol-fed rats. J Agric Food Chem 50:3597–601.

Kameoka S, Leavitt P, Chang C, Kuo S. 1999. Expression of antioxidant properties in human intestinal Caco-2 cells treated with dietary isoflavonoids. Cancer Lett 146:161–7.

Kapiotis S, Hermann M, Held I, Seelos C, Ehringer H, Gmeiner BM. 1997. Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. Arterioscler Thromb Vasc Biol 17:2868–74.

Kim JS, Kim JG, Kim WJ. 2004. Changes in isoflavone and oligosaccharides of soybeans during germination. Korean J Food Sci Technol 36:294–7.

Kraus C, Spiteller G, Mithöfer A, Ebel J. 1995. Quantification of glyceollins in non-elicited seedlings of *Glycine max* by gas chromatography-mass spectrometry. Phytochemistry 40:739–43.

Kudou S, Fleury Y, Welti D, Magnolato D, Uchida T, Ketamura K, Okubo K. 1991. Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). Agric Biol Chem 55:2227–33.

Lee CH, Yang L, Xu JZ, Yeung SYV, Huang Y, Chen ZY. 2005. Relative antioxidant activity of soybean isoflavones and their glycosides. Food Chem 90:735–41.

Lee JH, Renita M, Fioritto RJ, St Martin SK, Schwartz SJ, Vodovotz Y. 2004. Isoflavone characterization and antioxidant activity of Ohio soybeans. J Agric Food Chem 52:2647–51.

Liebler DC, Valcic S, Arora A, Burr JA, Cornejo S, Nair MG, Timmermann BN. 2001. Antioxidant reactions of green tea catechins and soy isoflavones. Adv Exp Med Biol 500:191–7.

Lin P-Y, Lai H-M. 2006. Bioactive compounds in legumes and their germinated products. J Agric Food Chem 54:3807–14.

Liu K-S. 1997. Biological and compositional changes during soybean maturation, storage, and germination. In: Liu K-S, editor. Soybeans: chemistry, technology, and utilization. New York: Chapman and Hall. p 129–30.

Marco GJ. 1968. A rapid method for the evaluation of antioxidants. J Am Oil Chem Soc 45:594–8.

Matthäus B. 2002. Antioxidant activity of extracts obtained from residues of different oilseeds. J Agric Food Chem 50:3444–52.

Messina MJ, Loprinzi CL. 2001. Soy for breast cancer survivors: a critical review of the literature. J Nutr 131:3095S–108S.

Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, Duthie GG. 1998. Antioxidant efficacy of phytoestrogens in chemical and biological systems. Arch Biochem Biophys 360:142–8.

Murphy PA, Barua K, Hauck CC. 2002. Solvent extraction selection in the determination of isoflavones in soy foods. J Chromatogr B 777:129–38.

Naim M, Gestetner B, Bondi A, Dirk Y. 1976. Antioxidative and antihemolytic activities of soybean isoflavones. J Agric Food Chem 2:1174–7.

Paxton JD. 1991. Biosynthesis and accumulation of legume phytoalexins. In: Sharma RP, Salunkhe DK, editors. Mycotoxins and phytoalexins. Boca Raton, Fla.: CRC Press. p 485–500.

Piubelli GC, Hoffmann-Campo CB, DeArruda IC, Franchini JC, Lara FM. 2003. Flavonoid increase in soybean as a response to *Nezara viridula* injury and its effect on insect-feeding preference. J Chem Ecol 29:1223–33.

Pratt DE, Birac PM. 1979. Source of antioxidant activity of soybeans and soy products. J Food Sci 44:1720–2.

Pyo Y-H, Lee T-C, Lee Y-C. 2005. Effect of lactic acid fermentation on enrichment of antioxidant properties and bioactive isoflavones in soybean. J Food Sci 70:215–20.

Rice-Evans CA, Miller NJ, Paganga G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol Med 20:933–56.

Rimbach G, De Pascual-Teresa S, Ewins BA, Matsugo S, Uchida Y, Minihane AM, Turner R, Vafeiadou K, Weinberg PD. 2003. Antioxidant and free radical scavenging activity of isoflavone metabolites. Xenobiot 33:913–25.

Ruiz-Larrea M, Mohan A, Paganga G, Miller N, Bolwell G, Rice-Evans C. 1997. Antioxidant activity of phytoestrogenic isoflavones. Free Radic Res 26:63–70.

Salvo VA, Boué SM, Fonseca JP, Elliott S, Corbitt C, Collins-Burow BM, Curiel TJ, Shih BY, Carter-Wientjes C, Wood CE, Erhardt P, Beckman B, McLachlan JA, Cleveland TE, Burow ME. 2006. Antiestrogenic glyceollins suppress human breast and ovarian carcinoma tumorigenesis. Clin Cancer Res 12(23):7159–64.

Seshime Y, Juvvadi PR, Fujii I, Kitamoto K. 2005. Discovery of a novel superfamily of type III polyketide synthases in *Aspergillus oryzae*. Biochem Biophys Res Commun 331:253–60.

Taga MS, Miller EE, Pratt DE. 1984. Chia seeds as a source of natural lipid antioxidants. J Am Oil Chem Soc 61:928–31.

Variyar PS, Limaye A, Sharma A. 2004. Radiation-induced enhancement of antioxidant contents of soybean (*Glycine max* Merrill). J Agric Food Chem 52:3385–8.

Wang C, Ma Q, Pagadala S, Sherrard MS, Krishnan PG. 1998. Changes of isoflavones during processing of soy protein isolates. J Am Oil Chem Soc 75:337–41.

Wang H-J, Murphy PA. 1996. Mass balance study of isoflavones during soybean processing. J Agric Food Chem 44:2377–83.

Wood CE, Clarkson TB, Appt SE, Franke AA, Boué SM, Burow ME, McCoy T, Cline JM. 2006. Effects of soybean glyceollins and estradiol on postmenopausal female monkeys. Nutrition Cancer 56(1):67–75.

Wu AH, Ziegler RG, Horn-Ross PL, Nomura AMY, West DW, Kolonel LN, Rosenthal F, Hoover RN, Pike MC. 1996. Tofu and risk of breast cancer in Asian-Americans. Cancer Epi Biomarkers Prev 5:901–6.

Zeringue HJ Jr. 1984. The accumulation of five fluorescent compounds in the cotton leaf induced by cell-free extracts of *Aspergillus flavus*. Phytochemistry 23:2501–3.

Zhu D, Hettiarachchy NS, Horax R, Chen P. 2005. Isoflavone contents in germinated soybean seeds. Plant Foods Human Nutr 60:147–51.